AWARD NUMBER: W81XWH-14-1-0398

TITLE: Targeting CD81 to Prevent Metastases in Breast Cancer

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REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE October 2015	**2. REPORT TYPE** Annual	**3. DATES COVERED** 30 Sep 2014 - 29 Sep 2015

4. TITLE AND SUBTITLE

Targeting CD81 to Prevent Metastases in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0398

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

None

14. ABSTRACT

During the research period, we tested a role for CD81 in breast cancer metastases and found that loss of CD81 expression in breast cancer cells impairs the number of circulating tumor cells. The experiments were performed using a protocol that we standardized for detection of circulating tumor cells in an immunocompetent syngeneic mouse model of breast cancer using FASTcell™ system.

15. SUBJECT TERMS

Breast cancer metastases, CD81, Circulating Tumor Cells (CTCs)

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	14	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

The abstract in Block 14 **must** state the purpose, scope, major findings and be an **up-to-date** report of the progress in terms of results and significance. Subject terms are keywords that may have been previously assigned to the proposal abstract or are keywords that may be significant to the research. The number of pages shall include all pages that have printed data (including the front cover, SF 298, table of contents, and all appendices). Please count pages carefully to ensure legibility and that there are no missing pages as this delays processing of reports. **Page numbers should be typed: please do not hand number pages.**

TABLE OF CONTENTS: Here is a sample table of contents in Microsoft Word format (25 KB) and in PDF (9 KB).

The text of the report must include all sections addressed in the table of contents to include the following. **DO** include the bolded section headings, but **DO NOT** include the *italicized* descriptions of section contents in your submitted reports.

1. **INTRODUCTION:**

The overarching hypothesis of our research work is that, CD81 (also known as target of the antiproliferative antibody 1, TAPA-1, or Tetraspanin-28), a transmembrane protein and a member of the tetraspanin family, is actively involved in the development of metastases in breast cancer and that blocking it may decrease the development and/or spread of metastases. Using syngeneic breast cancer tumor models, implantation of human breast cancer lines and patient derived xenografts in immunocompromised

mice, and by employing state of the art circulating tumor cell (CTC) detection technology, we will determine whether CD81 is critically involved in the generation of CTCs and if it plays a key role in the development of breast cancer tumor metastases. We will then test the use of CD81 targeting antibodies to curb CTC generation and metastatic progression. We will also explore the role of myeloid derived suppressor cells (MDSC) in CD81 regulation of CTCs.

2. KEYWORDS:

CD81, breast cancer metastases, circulating tumor cells (CTCs), 4T1, SRI FASTcell™ (Stanford Research Institute fiber-optic array scanning technology), cytokeratin, CD45

- **ACCOMPLISHMENTS**
- **What were the major goals of the project?**

The major goal of the project for year 1 was to determine the role of both host and tumor-derived CD81 in CTC generation using different syngeneic murine models of breast cancer. Below we have outlined milestones and accomplishments pertinent to research activities in the lab of Partnering PI, Dr. Stefanie Jeffrey only (the syngeneic mouse studies were done by the Initiaing PI, Dr. Shoshana Levy; Dr. Jeffrey performed CTCs studies on the mouse blood received from Dr. Levy's lab). There were no significant changes in the major direction of the proposed work.

Major Task 1: Determine if the absence of CD81 affects CTC shedding and metastases

Subtask 1 (months 1-3): Implant the mouse breast cancer cell line, 4T1, in immunocompetent wild type (WT) and in CD81KO mice. 1) Measure shedding of CTCs in WT and CD81KO mice during tumor growth, 2) monitor appearance of metastases using in vivo luminescent live imaging, and 3) count lung metastases

Subtask 2 (months 4-8): Determine if CD81 deficiency affects perioperative CTC shedding. Measure CTC shedding before and after mouse mastectomy. Implant the mouse breast cancer cell line, 4T1, in immunocompetent WT and in CD81KO mice. 1) Perform mouse mastectomies, 2) Measure CTC shedding before and after mouse mastectomy in WT and CD81KO mice.

Progress for Subtasks 1, 2 – We encountered two major, unanticipated hurdles during the completion of experiments outlined in subtask 1 and 2. (A) We had to spend considerable time, much more than what we had expected, in selecting the CTC capture platform to be used. We also tested the use of Vortex platform, another technology routinely used in our lab for CTC detection. Vortex microfluidically isolates live CTCs based on size criteria (CTCs are usually larger that WBCs and RBCs, so separation of CTCs from whole blood is straight forward and does not require RBC lysis. However, although our previous work (before this grant started) with human CTCs and CTCs from MDA-MB-231 mouse blood showed that the Vortex system was a good platform to use with these models or with human specimen, our testing of the Vortex platform using 4T1 cells, some of which are smaller than other cells lines, showed that Vortex insufficiently captured spiked 4T1 cells.

We then used the FASTcell™ technology to detect CTCs in the 4T1 syngeneic breast cancer model. This required RBC lysis optimization experiments and adpating cytokeratin staining protocols for 4T1 cells in FASTcell™. We have now successfully overcome hurdles and have now standardized the use of FASTcell™ for accurately testing murine 4T1 CTCs. Detailed results from our experiments are outlined in the sections below. (B) Although breeding of CD81 knockout mice to generate adequate number of mice for testing in all proposed experiments took more time than initially anticipated (performed by Dr. Levy's lab), this problem has also been resolved. We now have adequate numbers of knockout animals to successfully complete the work proposed in Subtasks 1, 2. We also have standardized and adapted the protocol for processing samples using the FASTcell™ protocol to accurately enumerate and characterize CTCs in blood from the syngeneic animal models. Experiments detailed in the subtasks 1, 2 are currently underway and will be completed shortly.

Subtask 4 (months 8-12): Determine the effect of CD81 knockdown (KD) in 4T1 tumor cells on their ability to metastasize in WT and CD81KO host mice. Knock-down CD81 in 4T1 cells using shRNA. Implant 4T1CD81KD cells in WT and CD81KO mice. 1) Measure shedding of CTCs in WT and CD81KO mice during tumor growth, 2) monitor appearance of metastases using in vivo luminescent live imaging, and 3) count lung metastases.

Progress for Subtask 4 **–** We have successfully tested the role for CD81 in generation of CTCs. By comparing the number of circulating tumor cells from mice implanted with either 4T1 tumor cells in which CD81 was knocked down (CD81KO) or wild type 4T1 cells (WT), we discovered that loss of CD81 expression diminishes CTC generation. This subtask at the month 12 of the funding period is almost 100% complete.

- **What was accomplished under these goals?**

Our key objective for the first year of funding was to test whether CD81 was involved in generation of circulating tumor cell in a syngeneic mouse model of breast cancer.

Key accomplishments during year one of the funding period are,

1) Having learned that the Vortex technology we were using did not adequately capture CTCs from 4T1 model systems, we systematically tested and adapted state of the art fiber-optic array scanning technology (FASTcell™) for detecting and characterizing circulating tumor cells in a syngeneic animal model (4T1) of breast cancer metastases. This is the first standardization of the FASTcell™ platform for analyzing CTCs in a syngeneic breast cancer model.
2) Using our newly established protocol involving FASTcell™, we have addressed the primary objective for year one of the study and demonstrated a potential role for tumor-derived CD81 in generating circulating tumor cells.

Results from studies completed in year one

1. Steps involved in sample processing for FASTcell™ technology do not affect CTC detection: A key step in preparing samples for FASTcell™ technology involves lysing the red blood cells (RBCs)

present in the whole blood. To ensure that ACK (Ammonium-Chloride-Potassium) Lysing Buffer used in RBC lysis doesn't affect staining of 4T1 cells, we treated 4T1 cell suspension with ACK buffer, washed, stained the samples for cytokeratin (CK, marker for cells of epithelial origin such as breast cancer cells) and processed the samples in the FASTcell™ platform. Our results demonstrate that accurate detection of CK in tumor cells is unaffected by sample processing.

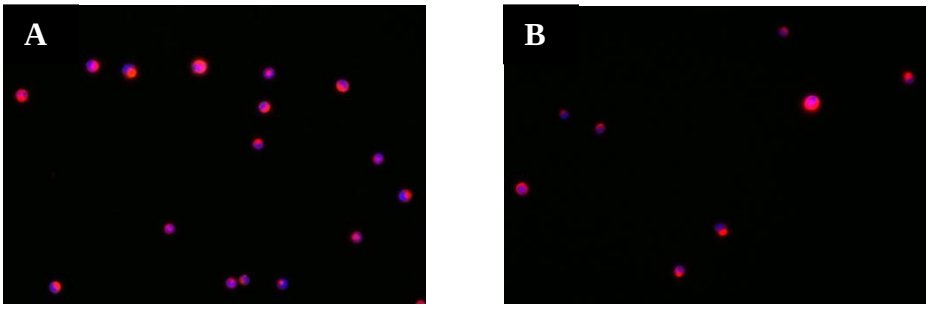
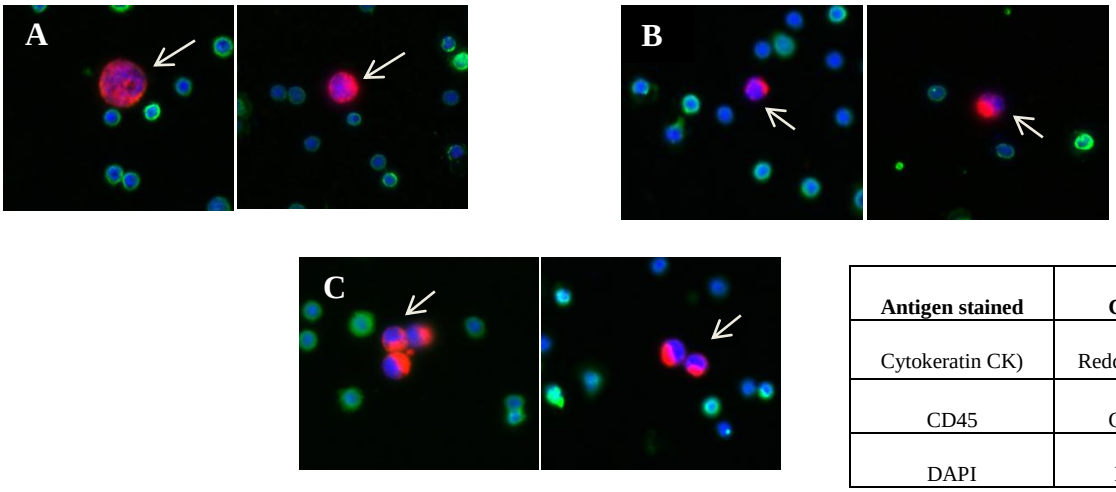


Figure 1. Cytokeratin positive 4T1 tumor cells (pink) in (A) untreated sample, and (B) sample subjected to ACK lysis treatment. Note the intensity of cytokeratin is unchanged between the two samples.

2. FASTcell™ technology accurately captures heterogeneous 4T1 tumor cell populations: Circulating tumor cells are heterogeneous and several reports have now demonstrated that often CTCs can be found as single cells, cluster of cells, cells of varying sizes, etc. To ensure that FASTcell™ can detect different 4T1 tumor cell populations, we spiked whole blood from BALB/c mice with 4T1 cells, processed the samples, stained for cytokeratin (CK), CD45 - to identify immune cells populations, and DAPI (nuclear stain). Imaged the stained samples looking specifically for CK^+, $CD45^-$ tumor cells.



Antigen stained	Color
Cytokeratin CK)	Reddish pink
CD45	Green
DAPI	Blue

Figure 2. Different tumor populations were clearly identified by our CTC detection platform. CK^+ and $CD45^-$ 4T1 tumor cells included (A) large cells, (B) small cells, and (C) cluster of tumor cells were all easily detected by FASTcell™ system. Arrows point to cytokeratin positive (CK^+, $CD45^-$) tumor cells. In

the background large numbers of $CD45^+$ (green color) immune cells are seen. The blue color seen in the images is the nuclear stain DAPI. All images presented above are at 20X magnification.

3. Determining the detection efficiency of FASTcell™ technology for 4T1 cells: Capturing tumor cells in small quantities of blood like in the case of mouse where usually no more than 200µl of sample is available presents an enormous technical challenge. Nevertheless, to ensure that we are efficiently capturing CTCs, we performed a series of experiments. Briefly, to 200µl of mouse blood (provided by Initiating PI - Dr. Shoshana Levy's lab) was spiked with varying numbers of 4T1 tumor cells. Individual blood samples added with known number of tumor cells were then treated with ACK RBC lysing buffer, stained for cytokeratin and CD45 using our standardized protocol and then probed for tumor cells present in each sample using the FASTcell™ platform. Based on the known number of cells we added and the number of cells we detect after processing, we then calculated from individual samples, the detection efficiency. We then performed a separate set of experiments wherein different numbers of either 4T1 cells expressing luciferase (WT) or 4T1 cells expressing luciferase in which CD81 has been knocked down (CD81KO) using CRIPSR-Cas9 technology were added to whole blood from mouse and then processed to calculate detection efficiency. Note – The validation experiments that we have done for using the FASTcell™ platform is not routinely performed for many CTC isolation technologies. We have invested a lot effort in ensuring that we have a reliable, robust work flow for detecting CTCs. This is very critical for the overall success of the grant.

Sample ID	Number of 4T1 cells added	Number of 4T1 cells detected	Retention Rate
S1	100	32	32%
S2	50	21	42%
S3	25	8	32%
S4	12.5	3	24%
S5	6.2	1	15%
S6	0	0	NA
Average Retention Rate			**29%**
Standard deviation			**9.69%**

Table 1. Decreasing numbers of 4T1 cells were mixed with 200 µl of whole blood from mouse and then processed as per procedure described in section above. Results from individual tubes are shown in the above table.

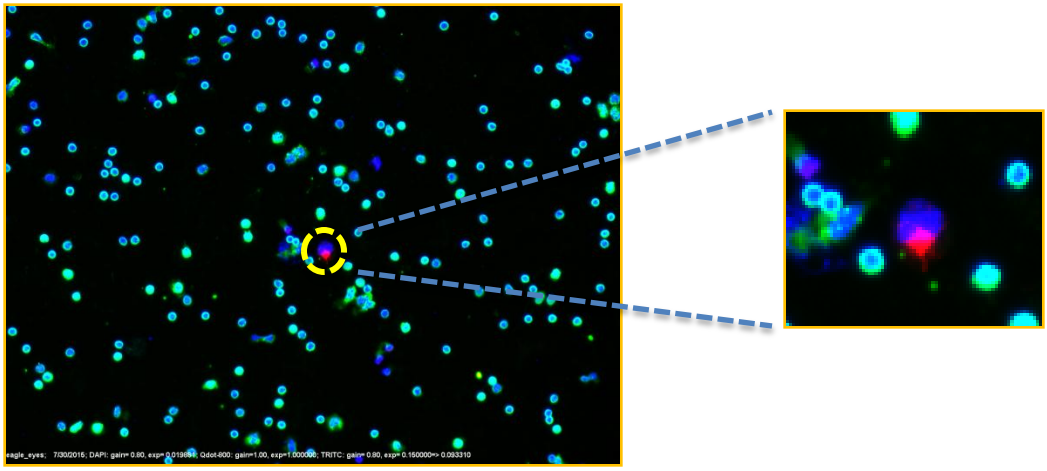
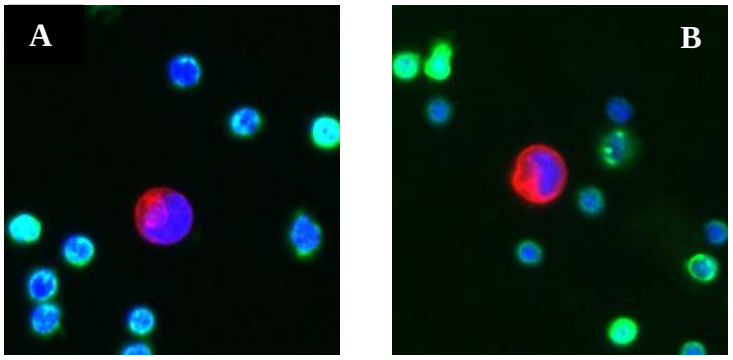


Figure 3. Stained image of a single tumor cell that was detected from the sample (S5) in Table 1 detected by FASTcell™ system. Left – Within the yellow dotted circle is the cytokeratin positive and ($CK^{+}CD45^{-}$) tumor cell. Right – the single tumor cell in the left image is cropped and magnified to demonstrate CK staining.

Well ID	Number of 4T1 WT cells added	Number of 4T1 WT cells detected	Detection Efficiency
S1	100	21	21%
S2	50	17	34%
S3	25	1	4%
S4	12.5	1	8%
S5	6.2	2	32%
S6	0	0	NA

Well ID	Number of 4T1 KO cells added	Number of 4T1 KO cells detected	Detection Efficiency
S7	100	117	117%
S8	50	35	70%
S9	25	18	72%
S11	12.5	7	56%
S12	6.2	0	0%
S13	0	0	NA

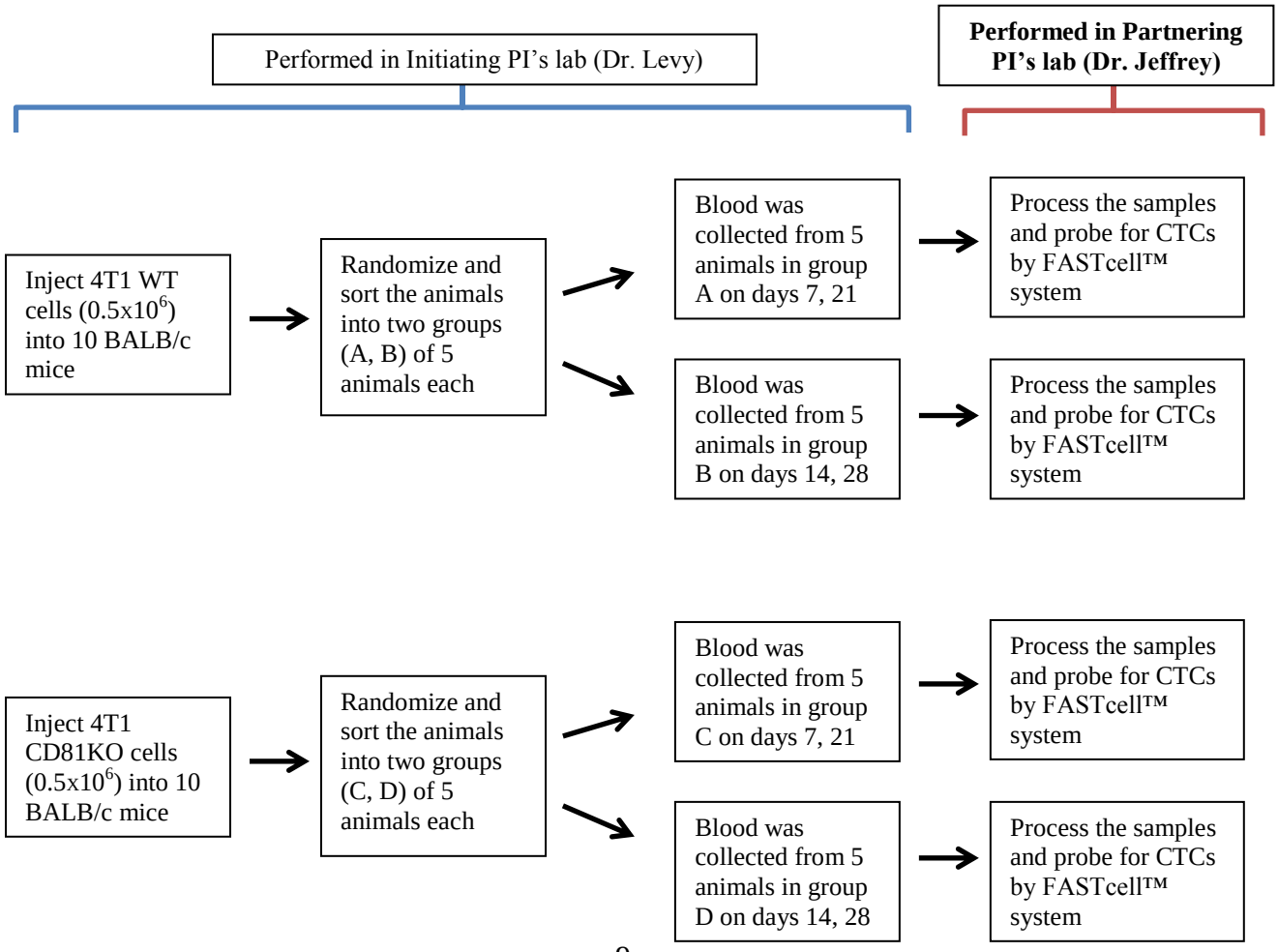
Table 2. Varying numbers of 4T1 WT or 4T1 KO cells were mixed with 200 µl of whole mouse blood and then processed as per procedure described in earlier sections. Results from individual tubes are shown in the above table.



Antigen stained	Color
Cytokeratin -CK	Reddish pink
CD45	Green
DAPI	Blue

Figure 4. Images of a tumor cells detected by presence of CK and absence of CD45 from mouse blood containing (A) 4T1 WT cells, and (B) 4T1 CD81KO cells as detected in the FASTcell™ platform.

Knocking down CD81 expression in 4T1 tumor cells impairs CTC generation: Using the above protocols that we standardized for detection of circulating tumor cells in the 4T1 syngeneic model, we then tested whether knocking down CD81 in tumor cells would curb the formation of CTCs. Briefly, 4T1 cells either WT or cells in which CD81 has been knocked down stably using CRISPR-Cas9 system were generated in the lab of Initiating PI. WT and CD81 KO cells were implanted subcutaneously near the mammary fat pad into individual BALB/c mice, sorted into different groups and blood was collected weekly via tail vein from different groups. TA the end of the experiment (Day 28) for group B and D apart from tail vein blood were also collected via cardiac puncture. The overall design of the experiment is elaborated below.

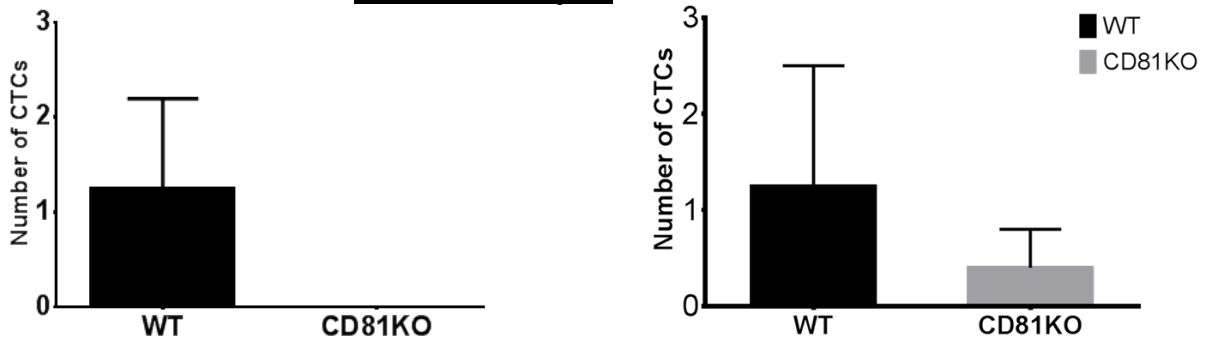


Results: Analyzing the number of CTCs in animals bearing either WT or CD81KO tumors revealed a clear difference between the two groups.

Tumors	Groups	Animal number	CTCs detected	
			Day 7	Day 21
WT	A	221	0	0
		222	0	0
		223	0	0
		224	0	0
		225	0	0
CD81 KO	C	206	0	0
		207	0	0
		208	0	0
		209	0	0
		210	0	0

Tumors	Groups	Animal number	CTCs detected		
			Day 14	Day 28 (tail vein)	Day 28 (cardiac puncture)
WT	B	946	1	0	0
		947	0	0	1
		948	0	dead	dead
		949	0	0	0
		950	0	5	4
CD81 KO	D	211	0	0	0
		212	0	2	0
		213	0	0	0
		214	0	0	0
		215	0	0	0

CTCs from Day 28



Graph of average number of CTCs (Day 28) from mice implanted with WT or CD81KO 4T1 cells. Graphs are Mean ± SEM. Blood used for sample analysis was collected via, left graph – cardiac puncture, right graph – tail vein.

Knocking down CD81 expression generates a unique CK+CD45+ circulating tumor cell population: We were concerned by the low number of CTCs we were detecting in the 4T1 syngeneic model. In our above protocol, we were using blood collected via tail vein from animals for CTC detection. To ensure that our route of blood collection was not affecting our results, on day 28 we collected blood from groups B and D (please refer to table above) via cardiac puncture as well and analyzed the samples for presence of CTCs. Analyzing blood obtained from cardiac puncture revealed that more animals in group B (bearing

WT tumors) had CTCs than animals in group D (bearing CD81KO tumors). Please refer to the graphs above. Interestingly two of the animals in the CD81KO group had high levels of a unique cytokeratin and CD45 double positive (CK+ CD45+) tumor population. Though one double positive cell population was detected from a single animal bearing WT tumor, animals bearing CD81 KO tumors clearly had higher numbers of this unique CK^+ $CD45^+$ cells. We do not fully understand the scope or importance of this discovery. We plan to test for presence of these double positive cells in our other models. Understanding the link between loss of CD81 and emergence of CK^+ $CD45^+$ cells in circulation could potentially yield several important clues regarding how CD81 regulates CTCs.

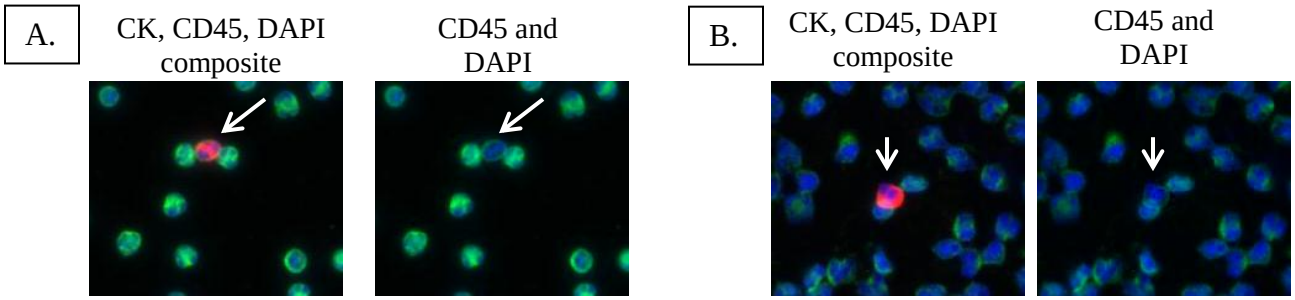
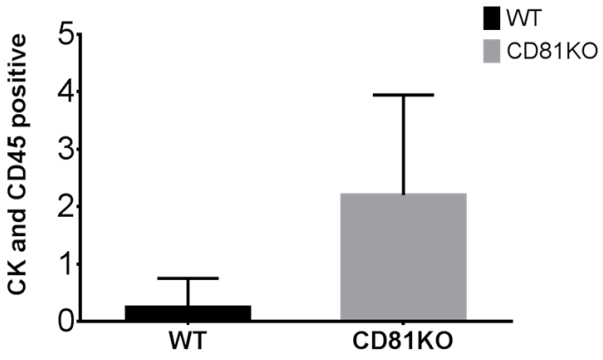


Figure 5. Images of cells stained for CK, CD45 and DAPI. In A, B - Left images reveal composite images of staining for CK, CD45, and DAPI. The corresponding images on the right side show only imaging for CD45 and DAPI from the same field of view. The arrows point to location of CK^+ and $CD45^+$ (double positive cells) as identified by the FASTcell™ system.

CK and CD45 double positive population



Graph of average number of CTCs (CK and CD45 double positive - Day 28) from mice implanted with WT or CD81KO 4T1 cells. Graphs are Mean ± SEM. Blood used for sample analysis was collected via cardiac puncture.

Tumors	Groups	Animal number	CK^+ and $CD45^+$ cells (Day 28 - cardiac puncture)
WT	B	946	0
		947	1
		949	0
		950	0
CD81 KO	D	211	0
		212	9
		213	0
		214	0
		215	2

- **What opportunities for training and professional development has the project provided?**

  A member of our lab who is performing all the experiments using the FASTcell™ technology received advanced training on the different protocols related to this technology from an expert at Stanford Research Institute (SRI).

- **How were the results disseminated to communities of interest?**

  Nothing to report

- **What do you plan to do during the next reporting period to accomplish the goals?**

  The experiments performed in year one of the funding period show a promising role for CD81 in regulating CTCs. Now that we have rigorously tested and standardized the protocol for CTC detection in the 4T1 syngeneic model, we are on track to finish the experiments outlined in subtasks1, 2 under the major task1 for year one at the earliest. The protocols we have standardized in year one will also enable us to expedite the completion of experiments under subtask 1 of major task2 for year 2. We have considerable prior experience working with CTCs from MDA MD 231 tumors growing in immunocompromised mice. All the protocols for detecting CTCs from mice implanted with MDA MB 231 cells have been standardized and therefore we plan to finish the experiments outlined in subtasks 2 and 3 for year two as planned without any delay. In our next year's report, we will be including results from subtasks 1, 2 from year one as well.

2. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**

  Detecting circulating tumor cells from immunocompetent (intact immune system) syngeneic mouse models of breast cancer is important, as the model systems we will use in years 2 and 3 will be performed with human cancer cells in immune incompetent mouse xenografts. For the time, in our present study, we have successfully addressed this issue. The protocol we have developed could be very useful in answering many questions related to biology of circulating tumor cells.

- **What was the impact on other disciplines?**

  Our protocol for detecting CTCs from syngeneic murine breast cancer model using FASTcell™ could potentially be adapted for syngeneic models in other cancers as well.

  What was the impact on technology transfer?

  Nothing to report

- **What was the impact on society beyond science and technology?**

  Nothing to report – too early in study

3. **CHANGES/PROBLEMS:** *:*

   Nothing to report

4. **PRODUCTS:**

- **Publications, conference papers, and presentations**

  Nothing to report
- **Journal publications.**
- Nothing to report
- **Books or other non-periodical, one-time publications.**
- Nothing to report
- **Other publications, conference papers, and presentations.** Nothing to report
- **Website(s) or other Internet site(s)**

  Nothing to report
- **Technologies or techniques**

  Nothing to report
- **Inventions, patent applications, and/or licenses**

  Nothing to report
- **Other Products**

  Nothing to report

5. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	*Stefanie Jeffrey*
Project Role:	*Partnering PI*
Researcher Identifier (e.g. ORCID ID):	------
Nearest person month worked:	*1.8 (no change)*
Contribution to Project:	*Designed and directed the project*
Funding Support:	*No change*
Name:	*Rakhi Gupta (replacing Kyra Heirich, non-key personnel)*
Project Role:	*Research Associate*
Researcher Identifier (e.g. ORCID ID):	------
Nearest person month worked:	*2.4 (no change)*
Contribution to Project:	*Performed the CTC-related work*
Funding Support:	*No change*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Nothing to report
- **What other organizations were involved as partners?**
- **Organization Name:** Stanford Research Institute (SRI)

- **Location of Organization:** Palo Alto, California
- **Partner's contribution to the projec**t
- **Financial support; -** Fee for service
- **In-kind support** - Nothing to report
- **Facilities** - We use the FASTcell™ system at Stanford Research Institute.
- **Collaboration** - We collaborate with Dr. Lidia Sambucetti and Dr. Xiaohe Liu at SRI
- **Personnel exchanges** - Nothing to report

6. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:**

  A separate report is being submitted by the Initiating PI

- **QUAD CHARTS:** Not applicable

7. APPENDICES: None